Arch Environ Contam Toxicol (2012) 62:233–242
DOI 10.1007/s00244-011-9705-8

Acute Toxicity of Nitrate and Nitrite to Sensitive Freshwater Insects, Mollusks, and a Crustacean

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Received: 5 May 2011 / Accepted: 16 August 2011 / Published online: 30 August 2011

Abstract Both point- and nonpoint-sources of pollution have contributed to increased inorganic nitrogen concentrations in freshwater ecosystems. Although numerous studies have investigated the toxic effects of ammonia on freshwater species, relatively little work has been performed to characterize the acute toxicity of the other two common inorganic nitrogen species: nitrate and nitrite. In particular, to our knowledge, no published data exist on the toxicity of nitrate and nitrite to North American freshwater bivalves (Mollusca) or stoneflies (Insecta, Plecoptera). We conducted acute (96-h) nitrate and nitrite toxicity tests with two stonefly species (*Allocapnia vivipara* and *Amphinemura delosa*), an amphipod (*Hyalella azteca*), two freshwater unionid mussels (*Lampsilis siliquoidea* and *Megalonaias nervosa*), a fingernail clam (*Sphaerium simile*), and a pond snail (*Lymnaea stagnalis*). Overall, we did not observe a particularly wide degree of variation in sensitivity to nitrate, with median lethal concentrations ranging from 357 to 937 mg NO_3-N/l; furthermore, no particular taxonomic group appeared to be more sensitive to nitrate than any other. In our nitrite tests, the two stoneflies tested were by far the most sensitive, and the three mollusks tested were the least sensitive. In contrast to what was observed in the nitrate tests, variation among species in sensitivity to nitrite spanned two orders of magnitude. Examination of the updated nitrite database, including previously published data, clearly showed that insects tended to be more sensitive than crustaceans, which were in turn more sensitive than mollusks. Although the toxic mechanism of nitrite is generally thought to be the conversion of oxygen-carrying pigments into forms that cannot carry oxygen, our observed trend in sensitivity of broad taxonomic groups, along with information on respiratory pigments in those groups, suggests that some other yet unknown mechanism may be even more important.

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The global nitrogen cycle has been substantially altered by anthropogenic activity, particularly through food and energy production (Galloway and Cowling 2002; Vitousek et al. 1997).Both point- and nonpoint-sources of pollution contribute to increased inorganic nitrogen concentrations in freshwater ecosystems. Point-sources include livestock and aquaculture operations, municipal and industrial sewage effluents, and runoff from other industrial activities. Nonpoint-sources tend to be associated with agriculture (i.e., fertilization, manure) and urbanization (i.e., runoff from septic systems, sewage) among other sources (reviewed by Camargo and Alonso 2006).The most abundant form of anthropogenic inorganic nitrogen in freshwaters is nitrate (NO_3^-), whereas ammonium (NH_4^+) and nitrite (NO_2^-) tend to account for a much smaller fraction of this pool (Stanley and Maxted 2008).

Ammonia toxicity to freshwater species has been widely studied (United States Environmental Protection Agency [USEPA] 2009), and the effects of nitrite on freshwater species, particularly in aquaculture settings, have been investigated (Gutzmer and Tomasso 1985; Jayasankar and Muthu 1983; Jensen 1990; Thurston et al. 1978; Tucker and Schwedler 1983). However, surprisingly few studies (reviewed in Camargo et al. 2005; Camargo and Alonso 2006) have produced acute nitrite or nitrate toxicity data in a manner generally following methods outlined by the American Society for Testing and Materials (ASTM 2002) and therefore can be considered for use in development of water-quality criteria by the USEPA. In the case of nitrate, this is probably a result of the fact that problems, such as eutrophication, tend to occur at nitrate concentrations that are much lower than those thought to be acutely toxic to freshwater vertebrates and invertebrates (Camargo and Alonso 2006). Nevertheless, in at least one case, water-quality criteria for protection of aquatic life are being developed for nitrate (Monson 2010). Based on this fact and the paucity of studies investigating nitrate or nitrite acute toxicity to freshwater invertebrates, the goal of this study was to begin to fill those data gaps.

Data on nitrate and nitrite sensitivity exist in the literature for a number of fish species, but few native North American invertebrate species have been tested with either compound. Freshwater unionid mussels have been shown to be among the most sensitive species to ammonia (Newton and Bartsch 2007; Newton et al. 2003; USEPA 2009; Wang et al. 2007a, b), and a recent study examined the toxicity of nitrate to several European mussel species (Douda 2010).However, with the exception of the exotic *Corbicula manilensis* (Chandler and Marking 1979),to our knowledge no published nitrate or nitrite toxicity data exist for North American bivalve mollusks. Therefore, we included two unionid mussels (*Lampsilis siliquoidea* and *Megalonaias nervosa*) and a fingernail clam (*Sphaerium simile*) as test species. Because we only had enough *M. nervosa* individuals to complete one test (nitrate), we substituted the pond snail (*Lymnaea stagnalis*) as an additional mollusk for nitrite testing. To date, caddisflies (Trichoptera) and mayflies (Ephemeroptera) are among the most sensitive species tested with nitrate and nitrite, respectively (Camargo and Ward 1992; Camargo et al. 2005; Kelso et al. 1999), so we chose representatives from another sensitive insect order, the stoneflies (Plecoptera; *Allocapnia vivipara* and *Amphinemura delosa*). No stoneflies have been tested previously for sensitivity to nitrate or nitrite. We also tested the amphipod *Hyalella azteca* because two European amphipod species (*Echinogammarus echinosetosus* and *Eulimnogammarus toletanus*) are among the most sensitive species tested. Published data on nitrate toxicity exist for *H. azteca* (Pandey et al. 2011), but we are unaware of published nitrite data. Finally, we collated published data on nitrate and nitrite toxicity to invertebrates (including the data presented here) into species mean acute values (SMAVs) to investigate potential trends in sensitivity among major invertebrate taxonomic groups.

Materials and Methods

Culture, Collection, and Holding of Test Organisms

Amphipods *H. azteca were* cultured in-house (Soucek Laboratory, Illinois Natural History Survey [INHS]) according to USEPA methods with modifications (USEPA 2000).Amphipods were cultured in a reconstituted laboratory water developed by Borgmann (1996; described further in later text) at 25°C and a 16 to 8-h (16:8) light-to-dark [L:D]) photoperiod and were fed TetraMin (TetraWerke, Melle, Germany) flake food. Nitex mesh was used as a substrate for the organisms. Organisms were cultured in the same water in which they were tested (detailed in later text) for at least two generations, and young were acclimated to the test temperature of 23°C during the course of a week before testing.

The stoneflies *A. vivipara* (Capniidae) and *A. delosa* (Nemouridae) were field-collected in December 2008 through January 2009 from Stony Creek, near Muncie, IL (Vermilion County) and in April 2009 from an unnamed tributary of the Vermilion River near Westville, IL (Vermilion County), respectively. Stoneflies were collected as later-instar nymphs. Stoneflies were returned to the laboratory in site water and were acclimated to laboratory conditions for $\sim$2 weeks; temperature was gradually adjusted (1°C/day) to a test temperature of 12 $\pm$ 1°C, and 50% of the water was changed every third day until holding water was 100% moderately hard reconstituted water (MHRW; USEPA 2002).The stoneflies were held in 6-l aquaria with a photoperiod of 16:8 L:D. Before testing, stoneflies were fed maple leaves that were collected from their respective collection sites and rinsed with deionized water. Other details of stonefly holding conditions followed recommendations of ASTM E729 (2002).

Fingernail clams (*S. simile*) were field-collected in June 2009 from Spring Creek, near Loda, IL (Iroquois County). Clams were collected as adults, returned to the laboratory (at INHS, Champaign, IL) in site water, and shortly thereafter allowed to release juveniles from their brood chambers in the laboratory. Testing was conducted with juveniles that were gradually acclimated to laboratory conditions for $\sim$2 weeks. Twenty percent of the water was changed daily until holding water was 100% MHRW; afterward, 50% of the water was changed daily. The temperature of the clam-holding water was gradually adjusted (1°C/day) from the water temperature at the time of collection to a test temperature of 23 $\pm$ 1°C. The clams were held in 6-L aquaria with a photoperiod of 16:8 L:D. Before testing, clams were fed daily a suspension of the green alga (*Ankistrodesmus falcatus*) at a rate of 1.25 mg (dry weight)/g clam (wet weight). Other details of clam-holding conditions followed recommendations of ASTM E729 (2002).

The freshwater mussels (*L. siliquoidea* and *M. nervosa*) were obtained from the culture facility of M. C. Barnhart at Missouri State University, Springfield, MO, and the Genoa Fish Hatchery, United States Fish and Wildlife Service, Genoa, WI, respectively. Both species were shipped as

Table 1 Salt concentrations (mg/l) added to deionized water for generation of dilution waters used for nitrate and nitrite toxicity testing with freshwater species

Water name	KCl	NaHCO3	MgSO4 (an)	CaSO4 (an)	CaCl2	NaBr
MHRW[a]	4	96	60	60	0	0
Borgmann[b]	4	84	30	0	111	1
ASTM hard[c]	8	192	120	120	0	0

an anhydrous salt used

[a] Used for tests with *S. simile*, *A. vivipara*, *A. delosa*, *L. siliquoidea*, *M. nervosa*

[b] Used for tests with *H. azteca*

[c] Used for tests with *L. stagnalis*

juveniles shortly after dropping from fish hosts and on receipt were placed in a mixture (~50:50 ratio) of the water in which they were shipped plus MHRW. Eight hours after receipt, a 50% water change was conducted to further acclimate the mussels to laboratory conditions. Mussels were received in water at close to test temperature, so extensive temperature acclimation was not required. Extended acclimation was not possible because of the need to conduct toxicity tests with <5-day-old juveniles (ASTM 2006).Mussels were held in 1-l beakers with gentle aeration and fed a mixture of Shellfish Diet 1800 and Nanno 3600 (Reed Mariculture, Campbell, CA) in the manner described in Wang et al. (2007b): 1 ml Nanno 3600 and 2 ml Shellfish Diet 1800 were added to 1.8 l MHRW, and 1 ml of this mixture was added per 300 ml of water in the holding vessels. Other details of mussel-holding conditions followed recommendations of ASTM 2455-06 (2006).

Pond snails (*L. stagnalis*) were obtained as egg masses from laboratory cultures at the United States Geological Survey's Columbia Environmental Research Center in Columbia, MO. On arrival, egg masses were placed in "ASTM hard water" (ASTM 2002) at 20°C with a photoperiod of 16:8 L:D and allowed to hatch. On hatching, young snails were fed organic lettuce rinsed with deionized water until testing (<7 days old). Other details of snail-holding conditions followed recommendations of ASTM E729 (2002).

Test Chemicals and Dilution Waters

The nitrate and nitrite sources for acute toxicity tests were sodium salts ($NaNO_3$, reagent grade, Chemical Abstracts Service [CAS] no. 7631-99-4, and $NaNO_2$, Certified American Chemical Society [ACS], CAS no. 7632-00-0; both from Fisher Scientific, Itasca, IL). We used different dilution waters depending on the species tested. Waters were formulated by adding a combination of four to five salts to distilled/deionized water (Table 1). Tests with *S. simile, A. vivipara, A. delosa, L. siliquoidea,* and *M. nervosa* were conducted using MHRW (USEPA 2002); tests with *H. azteca* were conducted with Borgmann (1996) water; and tests with *L. stagnalis* were conducted with ASTM hard water (ASTM 2002).

Acute Test Procedures

For *H. azteca, S. simile, A. vivipara*, *A. delosa,* and *L. stagnalis*, static, nonrenewal, acute toxicity tests were conducted according to guidelines detailed in ASTM E729-96 (2002), and for *L. siliquoidea* and *M. nervosa*, static, nonrenewal, acute toxicity tests were conducted according to guidelines detailed in ASTM 2455-06 (2006).Treatments were comprised of a 50% dilution series. Five to six concentrations were tested, with various reconstituted waters (Table 1) being used as both the diluents and control. Four replicates were tested per concentration, and five organisms were added to each replicate. The exception to this was *A. delosa* with only four organisms per replicate due to low availability. All tests had a duration of 96 h and a 16:8 L:D photoperiod. No organisms in the tests were fed or aerated. Tests with *H. azteca* and S. *simile* were conducted at 23 ± 1°C; *A. vivipara* and *A. delosa* were tested at 12 ± 1°C; the mussels and snails were tested at 20 ± 1°C. Test chambers for *A. vivipara* and *A. delosa* were 250-ml beakers; fingernail clams and snails were tested in 150-ml beakers; and the remaining species were tested in 50-ml beakers. To prevent escape of the snails, which breathe air, test chambers were covered with a synthetic mesh fabric. In most cases, early life stages of the species were used in acute tests: *H. azteca* were 7 to 14 days old; mussels were <5 days old; snails were <7 days old; and fingernail clams were juveniles (~2 weeks old). The stoneflies were later-instar nymphs. For *H. azteca*, *A. vivipara*, and *A. delosa*, nitex mesh was added to each test chamber to provide substrate for these benthic invertebrates. Percent survival in each replicate was recorded every 24 h and at the end of the exposure period. A dissecting microscope was used to assess survival of all species. At the end of 96-h tests, bivalves were transferred to clean dilution water with food for evaluation of survival; determinations of mortality and survival were made within 1 h after transfer to clean water. Individuals with undetectable foot movement or ciliary motion (determined using a dissecting scope) were considered dead. All median lethal concentration (LC_{50}) values were calculated using trimmed Spearman–Karber method (Hamilton et al. 1977).

Standard water-chemistry parameters—including temperature, pH, conductivity, dissolved oxygen, alkalinity and hardness—were measured at both the beginning and the end of each exposure period. pH measurements were made using an Accumet (Fisher Scientific, Pittsburgh, PA) model AB15 pH meter equipped with an Accumet gel-filled combination electrode (accuracy less than ±0.05 pH at 25°C). Dissolved oxygen was measured using an air-calibrated Yellow Springs Instruments (RDP, Dayton, OH) model 55 meter. Conductivity measurements were made using a Mettler Toledo (Fisher Scientific) model MC226 conductivity/TDS meter. Alkalinity and hardness were measured by titration as described by the American Public Health Association (2005). For one test, we measured ammonia to ensure that increased levels of this substance were not confounding our results. Ammonia was measured using a Thermo Orion 4 Star (Fisher Scientific) bench-top meter with an Orion model no. 9512 ammonia probe. At both the beginning and end of acute tests, water samples from each treatment were collected and submitted to the Water Quality Laboratory in the Department of Agricultural and Biological Engineering, University of Illinois at Urbana-Champaign, for confirmation of nitrate and nitrite concentrations according to USEPA methods 353.1 (USEPA 1978) and 354.1 (USEPA 1971). Both anions were measured in all tests to ensure that toxicity was attributable to the particular anion in question.

Calculation of SMAVs

We calculated SMAVs for all invertebrate species for which published data are available, including only LC_{50}s that came from tests generally conducted according to guidelines detailed in ASTM E729 (2002). In particular, we only included tests that were conducted for 48 or 96 h for cladocerans (96 h for other species) and tests in which organisms were not fed (ASTM 2002). In addition, we only included tests conducted with sodium salts of these two anions. In some cases, LC_{50}s reported were generated from nominal concentrations. In addition, some tests were conducted with adults or organisms of unknown age, but we indicate those instances. For data reported in units other than mg NO_3-N/l or mg NO_2-N/l we converted concentrations as follows: mg $NaNO_3$/l was converted to mg NO_3/l by multiplying by 0.729515; mg NO_3/l was converted to mg NO_3-N/l by multiplying by 0.225897; mg $NaNO_2$/l was converted to mg NO_2/l by multiplying by 0.666792; and mg NO_2/l was converted to mg NO_2-N/l by multiplying by 0.304457. In calculating SMAVs, we did not include tests in which no concentration produced sufficient mortality to calculate an LC_{50}; although in the case of *Pomacea paludosa* (Corrao et al. 2006) we calculated the geometric mean of the highest test concentration in four separate tests that produced insufficient mortality because no LC_{50}s were generated. Otherwise, SMAVs were calculated as the geometric mean of all available LC_{50}s for a given species. In many cases, only one LC_{50} is available for a given species, and this is reported as the SMAV even although it is not a mean.

Results

Nitrate Toxicity

For the 96-h nitrate toxicity tests, mean water temperatures remained within 1°C of targets; mean pH values ranged from 7.9 to 8.0; and hardness ranged from 90 to 117 mg/l as $CaCO_3$; there was low variability within tests (Table 2). Mean dissolved oxygen (DO) concentrations were within appropriate ranges for all tests at the various test temperatures (Table 2).Measured nitrate concentrations in test solutions were similar to nominal concentrations, with only minimal differences between samples from the beginning and the end of the test. The overall average (for all species) absolute value of the percent difference between nominal and measured NO_3-N concentrations was 3.33%. The range of these values for individual measurements for all tests was 0.1 to 12.7%. The maximum NO_2-N concentration observed in a nitrate toxicity test was 0.024 mg/l, but most measurements were lower than method detection limits. In the *M. nervosa* test, we measured total ammonia concentrations, and all treatments had concentrations lower than our lowest calibration standard (0.05 mg/l). In all six tests, control survival was at least 90%.

The 96-h LC_{50} values based on measured NO_3-N concentrations ranged from 357 (*L. siliquoidea*) to 937 mg/l for *M. nervosa* (Table 2). Although the highest and lowest LC_{50}s were for the two unionid mussels, the two stoneflies also had a wide range of sensitivities. *A. vivipara* was relatively insensitive with an LC_{50} of 836 mg NO_3-N/l, whereas *A. delosa* had a much lower LC_{50} (456 mg/l). The order of sensitivity (lowest to highest LC_{50}) was as follows: *L. siliquoidea* > *S. simile* > *A. delosa* > *H. azteca* > *A. vivipara* > *M. nervosa*.

Nitrite Toxicity

For the 96-h nitrite toxicity tests, mean water temperatures remained within 1°C of targets, mean pH values ranged from 7.7 to 8.3, and hardness ranged from 89 to 156 mg/l as $CaCO_3$, with low variability within tests (Table 3).As was the case for the nitrate tests, mean dissolved oxygen

Table 2 Ninety-six-hour NO_3-N LC_{50}s and measured water-quality conditions[a] for toxicity tests with six freshwater species

Species	Temp (SD) (°C)	pH (SD) (SU)	Hardness (SD) (mg/l as $CaCO_3$)	DO (mg)/l	LC50 (95% CI) (mg NO_3-N/l)
A. vivipara	11.0 (0.1)	7.9 (0.1)	99 (1)	10.3 (0.4)	836 (580–1206)
A. delosa	12.5 (0.2)	7.9 (0.0)	91 (1)	9.4 (0.5)	456 (325–642)
H. azteca	22.5 (0.2)	8.0 (0.0)	117 (7)	8.1 (0.1)	667 (559–742)
S. simile	22.8 (0.1)	8.0 (0.1)	90 (1)	7.3 (0.9)	371 (323–426)
L. siliquoidea	20.0 (0.1)	7.9 (0.0)	91 (1)	7.9 (0.1)	357 (250–509)
M. nervosa	20.9 (0.0)	8.0 (0.1)	91 (1)	8.2 (0.2)	937 (818–1073)

[a] Water-quality values are geometric means of measurements taken in all test concentrations throughout the duration of the test

Table 3 Ninety-six-hour NO_2-N LC_{50}s and measured water-quality conditions[a] for toxicity tests with six freshwater species

Species	Temp (SD) (°C)	pH (SD) (SU)	Hardness (SD) (mg/l as $CaCO_3$)	DO (mg)/l	LC50 (95% CI) (mg NO_3-N/l)
A. vivipara	11.5 (0.1)	7.9 (0.2)	99 (1)	9.9 (0.6)	1.5 (0.6–3.7)
A. delosa	12.4 (0.2)	7.9 (0.0)	90 (1)	9.5 (0.4)	1.0 (0.8–1.2)
H. azteca	22.7 (0.2)	7.9 (0.0)	117 (2)	8.1 (0.1)	12.5 (9.4–15.9)
S. simile	22.7 (0.1)	7.7 (0.3)	89 (1)	6.7 (0.7)	55.7 (43.0–72.1)
L. siliquoidea	20.0 (0.4)	7.9 (0.0)	89 (1)	7.7 (0.1)	176.5 (145–215)
L. stagnalis	20.3 (0.3)	8.3 (0.1)	156 (4)	8.4 (0.1)	55.8 (36.7–87.8)

[a] Water-quality values are geometric means of measurements taken in all test concentrations throughout the duration of the test

concentrations were within appropriate ranges for all tests at the various test temperatures (Table 3).Measured nitrite concentrations in test solutions were similar to nominal concentrations, with only minimal differences between samples from the beginning and the end of the test. The overall average (for all species) absolute value of the percent difference between nominal and measured NO_2-N concentrations was 2.91%. The range of these values for individual measurements for all tests was 0 to 7.4%. The highest mean NO_3-N concentration observed in a nitrite toxicity test was 6.93 mg/l, a concentration ~51-fold lower than the lowest LC_{50} observed. In all six tests, control survival was at least 90%.

The 96-h LC_{50} values based on measured NO_2-N concentrations ranged from 1.0 (*A. delosa*) to 176.5 mg/l for *L. siliquoidea* (Table 3).In contrast to the nitrate toxicity tests, there were clear differences in sensitivity among taxonomic groups, with the stoneflies being the most sensitive, followed by the crustacean, and then the three mollusks. The order of sensitivity (lowest to highest LC_{50}) was as follows: *A. delosa* > *A. vivipara* > *H. azteca* > *S. simile* > *L. stagnalis* > *L. siliquoidea*.

Discussion

With these results, we have nearly doubled the published data meeting the acceptability requirements listed previously on acute nitrate toxicity to freshwater invertebrates (Table 4). We have also substantially expanded the acute-nitrite toxicity database (Table 5). In particular, we believe these are the first published data on acute nitrate and nitrite toxicity to stoneflies, and to North American freshwater unionids and fingernail clams, with the latter being groups that have been shown to be extremely sensitive to ammonia exposure (USEPA 2009).

In the nitrate tests, there was moderate variability in sensitivity among the species we tested, particularly within the bivalve mollusks. The LC_{50}s for *L. siliquoidea* and *S. simile* were quite similar, but *M. nervosa* was much more tolerant, with an LC_{50} ~2.5-fold greater than that of the other two species. In previous work, *M. nervosa* was also much less sensitive than *L. siliquoidea* to boron exposure (Soucek et al. 2011). The two stonefly species (*A. vivipara* and *A. delosa*) also had relatively disparate nitrate LC_{50}s, although their 95% CIs overlapped. Camargo et al. (2005) suggested that nitrate concentrations do not frequently exceed 25 mg NO_3-N/l in surface waters, and all of our LC_{50}s were well above this concentration. In the nitrite tests, the LC_{50}s for the two stoneflies were nearly identical, as were those for the fingernail clam (*S. simile*) and the pond snail (*L. stagnalis*); however, the third mollusk (*L. siliquoidea*) had an LC_{50} more than 3-fold greater than those of the other two tested. Alonso and Camargo (2006) pointed out that nitrite concentrations may exceed 73 mg NO_2-N/l in polluted surface waters, and most of the LC_{50}s

Table 4 Freshwater invertebrate SMAVs for NO_3-N from the literature and the current study

Species(reference)	Taxonomic group	SMAV (mg NO3-N/l)	Life-stage tested
Echinogammarus echinosetosus[3]	Crustacean	63	Adult
Eulimnogammarus toletanus[3]	Crustacean	85	Adult
Hydropsyche occidentalis[2]	Insect	103 (97, 109)	Late-instar larvae
Cheumatopsyche petiti[2]	Insect	138 (114, 166)	Late-instar larvae
Hydropsyche exocellata[3]	Insect	270	Late-instar larvae
Hyalella azteca[6,8]	Crustacean	287 (124, 667)	Adults[6]; 7–10 days[8]
Lampsilis siliquoidea[8]	Mollusk	357	<5 days
Sphaerium simile[8]	Mollusk	371	<2 week
Ceriodaphnia dubia[7]	Crustacean	374 (374, 374)	<24 h
Daphnia magna[7]	Crustacean	447 (323, 453, 611)	<48 h
Amphinemura delosa[8]	Insect	456	Late-instar larvae
Allocapnia vivipara[8]	Insect	836	Late-instar larvae
Anodonta anatina[5]	Mollusk	922	1–10 days
Megalonaias nervosa[8]	Mollusk	938	<5 days
Potamopyrgus antipodarum[1]	Mollusk	1042	Unknown
Unio crassus[5]	Mollusk	1272	1–10 days
Pomacea paludosa[4]	Mollusk	>197 (all >)	Adults and juveniles

All SMAVs are median lethal concentrations (LC_{50}s) for 96-h tests except for those for *Ceriodaphnia* and *Daphnia* (48-h). When more than one test is included in a SMAV, individual test results are provided in parentheses; the SMAV is the geometric mean of the individual tests

[a] Formula used to convert mg $NaNO_3$/l to mg NO_3/l: $[NO_3] = [NaNO_3] \times 0.729515$

[b] Formula used to convert mg NO_3/l to mg NO_3-N/l: $[NO_3\text{-N}] = [NO_3] \times 0.225897$

[1] Alonso and Camargo (2003), [2] Camargo and Ward (1992), [3] Camargo et al. (2005), [4] Corrao et al. (2006), [5] Douda (2010), [6] Pandey et al. (2011), [7] Scott and Crunkilton (2000), [8] Current study

generated in the present study were lower than this concentration.

For both nitrate and nitrite, the LC_{50}s for *H. azteca* in our study approximated the median among the species we tested. This is in contrast to the findings of Camargo et al. (2005) and Alonso and Camargo (2006), who found two amphipod species, *Echinogammarus echinosetosus* and *Eulimnogammarus toletanus*, to be among the most sensitive species tested thus far (Tables 4, 5). Our nitrate LC_{50} for *H. azteca* (tested as 7- to 14-day-old juveniles) was 8- to 10-fold greater than the LC_{50}s reported for the other two amphipods, which were tested as adults (Camargo et al. 2005),and the our nitrite LC_{50} was 5- to 6-fold greater (Alonso and Camargo 2006). In addition, the nitrate LC_{50} reported by Pandey et al. (2011) for adult *Hyalella azteca* is ~5-fold lower than that reported in the present study for juveniles. There are two potential explanations for these disparities, the first being simply a wide range of sensitivity among members of this order. *Echinogammarus* sp. and *Eulimnogammarus* sp. belong to the family Gammaridae, whereas *Hyalella* sp. belongs to the family Hyalellidae. In our study, we observed a 3-fold difference in nitrite sensitivity among members of the same family (Unionidae); therefore, physiological differences among members of different families could explain the disparity.

An alternative explanation for the disparity in results among these amphipod tests is the difference in chloride concentration in the test water. Other investigators have shown that chloride, bromide, and nitrite compete for the same uptake mechanism in crayfish (Jensen 1996; Harris and Coley 1991).Alonso and Camargo (2008)did follow-up work to show that increasing chloride concentration from 27 to 108 mg/l decreased percent mortality of *E. toletanus* exposed to 5.1 mg NO_2-N/l from ~90 to <20% after 96 h (LC_{50}s were not generated). Kozák et al. (2005)observed a strong positive linear relation between chloride concentration in test water and nitrite LC_{50} for the crayfish *Orconectes limosus*, with LC_{50}s ranging from 4.8 to 96.6 mg NO_2-N/l at chloride concentrations ranging from 11 to 400 mg/l. Similarly, previous work with *H. azteca* has indicated that increasing chloride concentration from 5 to 25 mg/l increased the 96-h sulfate LC_{50} by >3-fold (Soucek 2007).Chloride may also regulate nitrate toxicity, but to our knowledge this has not been tested. Alonso and Camargo (2006) and Camargo et al. (2005)did not report chloride concentration in their test water, but if it was the same as their base water with 27 mg/l in Alonso and Camargo (2008), chloride and bromide concentrations in dilution water might well explain the disparity between the responses of their amphipods and ours.

Table 5 Freshwater invertebrate SMAVs for NO2-N[a,b] from the literature and the current study

Species(reference)	Taxonomic group	SMAV (mg NO2-N/l)	Life-stage tested
Amphinemura delosa[13]	Insect	1.0	Late-instar larvae
Hexagenia sp.[8]	Insect	1.4	Late-instar larvae
Allocapnia vivipara[13]	Insect	1.5	Late-instar larvae
Procambarus simulans[3]	Crustacean	1.9	Unknown
Eulimnogammarus toletanus[2]	Crustacean	2.1	Unknown
Ephemerella sp.[8]	Insect	2.5	Late-instar larvae
Echinogammarus echinosetosus [2]	Crustacean	2.6	Unknown
Cherax quadricarinatus[10–12]	Crustacean	5.0 (1.0, 4.7, 25.9)	10,11 Juveniles; 12 hatchlings
Gammarus fasciatus[5]	Crustacean	6.5 (6.5, 6.5)	Juveniles
Procambarus clarkii[6,7]	Crustacean	7.1 (8.5, 5.9)	6 Unknown; 7 juveniles
Daphnia magna[5]	Crustacean	9.0 (8.3, 9.7)	Juveniles
Hyalella azteca[13]	Crustacean	12.5	7–10 days
Helisoma trivolvis[5]	Mollusk	15.6 (12.0, 20.3)	Juveniles
Asellus intermedius[5]	Crustacean	20.3[a]	Juveniles
Dugesia tigrina[5]	Platyhelminth	20.3[a]	Juveniles
Lumbriculus variegatus[5]	Oligochaete	20.3[a]	Juveniles
Orconectes limosus[9]	Crustacean	31.8 (5, 18, 35, 51, 74, 97)	1 year
Sphaerium simile[13]	Mollusk	55.7	<2 week
Lymnaea stagnalis[13]	Mollusk	55.8	<7 days
Polycelis felina[2]	Platyhelminth	60.0	Unknown
Lampsilis siliquoidea[13]	Mollusk	176.5	<5 days
Corbicula manilensis[4]	Mollusk	250.0	Unknown
Potamopyrgus antipodarum[1]	Mollusk	535.0	Unknown

All SMAVs are median lethal concentrations (LC_{50}s) for 96-h tests. When more than one test is included in a SMAV, individual test results are provided in parentheses; the SMAV is the geometric mean of the individual tests

Formula used to convert mg $NaNO_2$/l to mg NO_2/l: $[NO_2] = [NaNO_2] \times 0.666792$

Formula used to convert mg NO_2/l to mg NO_2-N/l: $[NO_2\text{-}N] = [NO_2] \times 0.304457$

[1] Alonso and Camargo (2003), [2] Alonso and Camargo (2006), [3] Beitinger and Huey (1981), [4] Chandler and Marking (1979), [5] Ewell et al. (1986), [6] Gutzmer and Tomasso (1985), [7] Hymel (1985), [8] Kelso et al. (1999), [9] Kozák et al. (2005), [10] Liu et al. 1995, [11] Meade and Watts 1995, [12] Rouse et al. 1995, [13] Current study. Reference 8 had LC_{50}s for *Asellus* sp., *Daphnia* sp., *Gammarus* sp., and *Polycelis* sp. that are not included in Table 5 because other references list species for these genera. LC_{50}s for those tests were 71.0, 18.0, 12.3, and 61.6 mg NO_2-N/l, respectively

[a] The same reference also reported an $LC_{50} > 20.3$ mg NO_2-N/l for this species

The test with *H. azteca* from Pandey et al. (2011)was conducted in USEPA (2000) MHRW, which has a nominal chloride concentration of 1.9 mg/l and no bromide (nominally). Our *H. azteca* tests were conducted in a reconstituted water developed by Borgmann (1996), which has a nominal chloride concentration of 72 mg/l and a bromide concentration of 0.8 mg/l. According to Borgmann (1996), bromide is a necessary ion for long-term survival of this species. The difference in chloride and bromide concentrations in the dilution waters used in these two studies may also account for the disparity in results. Furthermore, Kemble et al. (1999) observed high mortality rates in *H. azteca* tested for 28 days in reference sediments with MHRW used as overlying water and concluded that this reconstituted water did not support long-term survival, growth, and reproduction of this species. This is most likely a result of the low chloride concentration in this water. Therefore, although acceptable control mortality may be achieved in short-term exposures of *H. azteca* with MHRW, results may reflect those of stressed organisms.

Examining the distribution of SMAVs for nitrate and nitrite among the various taxonomic groups (Tables 4; 5), several points of interest are shown. The first point is that for acute nitrate toxicity (Table 4), no particular group appears to be more sensitive than any other. Crustaceans had LC_{50}s ranging from 63 to 667, insects ranged from 97 to 836, and mollusks ranged from 357 to 1272 mg NO_3-N/l. In the case of *P. paludosa*, four tests were conducted with a mean highest test concentration of 197 mg NO_3-N/l, and no test produced >10% mortality (Corrao et al. 2006).The lack of trend among broad taxonomic groups in sensitivity to nitrate may be a function of the relatively small number of species tested with this compound. Tests with more species are warranted.

Unlike the case of the nitrate SMAVs, there was a clear trend of sensitivity among taxonomic groups to nitrite (Table 5).Insects clearly ranked among the most sensitive species tested, crustaceans followed insects in sensitivity, and mollusks tended to be the least sensitive to this compound. Too few tests have been conducted with oligochaetes and platyhelminths to make observations on their sensitivity. This trend in sensitivity of insect > crustacean > mollusk is interesting on several levels. The first is the finding that mollusks, particularly unionid mussels and fingernail clams, are relatively insensitive to nitrite toxicity. This is of interest because these groups are the most sensitive, among numerous taxa tested, to the third common form of inorganic nitrogen: ammonia (USEPA 2009). Of the ~67 genera for which genus mean acute values are included in the draft calculation of the ammonia criterion maximum concentration by the USEPA (2009), 8 of the top 10 most sensitive genera were mollusks, and 6 of those were bivalves. In the case of nitrite, the most sensitive mollusk (*Helisoma*; Ewell et al. 1986) ranked 13th of 23, and the bivalves (*Sphaerium, Lampsilis*, and *Corbicula*) appear to be especially insensitive (Table 5).

The trend of bivalve mollusks being less sensitive to nitrite than crustaceans is not surprising when viewed in light of the purported toxic mechanism of this anion. Nitrite is primarily thought to cause toxicity by converting oxygen-carrying blood pigments, such as hemoglobin and hemocyanin, into forms that cannot carry oxygen, such as methemoglobin and methemocyanin (Camargo and Alonso 2006). Most crustaceans have hemocyanins as respiratory pigments (Thorp and Covich 2001), although some have hemoglobin (e.g., *Daphnia*; Sugano and Hoshi 1971). However, most freshwater bivalves do not have respiratory pigments in their hemolymph (McMahon and Bogan 2001). Clearly, the lack of respiratory pigments in freshwater bivalves removes the primary cause of nitrite toxicity.

This narrative becomes a bit more tenuous with the snails *Helisoma*, *Lymnaea*, and *Potamopyrgus*, which also were relatively insensitive to nitrite compared with insects and crustaceans. *Lymnaea* sp., like most freshwater gastropods, uses hemocyanin as a respiratory pigment (Hall et al. 1975), whereas *Helisoma* is in a unique family (Planorbidae) among snails that uses hemoglobin (Smith 2001).The presence of hemoglobin may explain the relative sensitivity of *Helisoma* among the mollusks, whereas perhaps hemocyanin of *Lymnaea* is less susceptible to the effects of nitrite than hemoglobin would be in a snail. However, *Potamopyrgus* belongs to a family (Hydrobiidae) that has hemocyanins (Smith 2001), and it was less sensitive than any of the bivalves, which have no respiratory pigments. Further confusing the issue is the fact that the most sensitive group of all are the insects (Table 5). With the exception of some members of the families Chironomidae and Notonectidae, freshwater insects do not use respiratory pigments but rather employ a tracheal system to supply their tissues with oxygen (Resh et al. 2008). Clearly some other mechanism must be playing a substantial role in nitrite toxicity to some groups of freshwater invertebrates, as has been suggested by Kelso et al. (1999). Further work should be performed to characterize this mechanism.

In conclusion, we have generated nitrite and nitrate LC_{50}s for a number of new species, including the first published data for freshwater bivalves and stoneflies. In our nitrate tests, the bivalves *S. simile* and *L. siliquoidea* were the most sensitive species tested, whereas a third bivalve, *M. nervosa*, was the least sensitive. Overall, we did not observe a particularly wide degree of variation in sensitivity to nitrate: LC_{50}s ranged from 357 to 937 mg NO_3-N/l. In our nitrite tests, the two stoneflies, *A. vivipara* and *A. delosa*, were by far the most sensitive, and the three mollusks tested were the least sensitive. Variation among species in sensitivity to nitrite spanned two orders of magnitude. Incorporating our data with the published literature, no clear trend in nitrate sensitivity among broad taxonomic groups was apparent; however, examination of the updated nitrite database showed a clear trend, with insects being more sensitive than crustaceans, which were in turn more sensitive than mollusks. Although the toxic mechanism of nitrite is generally thought to be due to the conversion of oxygen-carrying pigments into forms that cannot carry oxygen (Camargo and Alonso 2006), our observed trend in the sensitivity of broad taxonomic groups, along with information on respiratory pigments in those groups, suggests that the some other yet unknown mechanism may be even more important.

Acknowledgments This study was funded by USEPA Region 5 by way of Great Lakes Environmental Center. Kaley Major (INHS) provided technical assistance with collecting and culturing of organisms and conducting toxicity tests. Ed Dewalt (INHS) assisted with collection and identification of stonefly nymphs. Duane Kimme (UIUC) measured nitrate and nitrite concentrations in our water samples. Comments from two anonymous reviewers greatly improved this manuscript.

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